Educational Module

Title:

A Scientific Investigation on Alcohol Fermentation and Biomass Conversion

Author:

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Grade Level/Subject:

AP (Advanced Placement) Biology
Grades 11 and 12
90 minute periods

Curriculum Standard:

AAAS Benchmarks. Project 2061.
Section 1, The Nature of Science.
By the end of the 12th grade, students should know that

- **1B Scientific Inquiry:** Hypotheses are widely used in science for choosing what data to pay attention to and what additional data to seek, and for guiding the interpretation of the data (both new and previously available).

Section 5, The Living Environment.
By the end of 12th grade, students should know that

- **5C Cells:** Complex interactions among the different kinds of molecules in the cell cause distinct cycles of activities, such as growth and division. Cell behavior can also be affected by molecules from other parts of the organism or even other organisms.

Section 9, The Mathematical World.
By the end of 12th grade, students should know that

- **9B Symbolic Relationships:** In some cases, the more of something there is, the more rapidly it may change (as the number of births is proportional to the size of the population). In other cases, the rate of change of something depends on how much there is of something else (as the rate of change of speed is proportional to the amount of force acting).
- **9B Symbolic Relationships:** Tables, graphs, and symbols are alternative ways of representing data and relationships that can be translated from one to another.

Section 11, Common Themes.
By the end of 12th grade, students should know that

- **Constancy and Change:** Graphs and equations are useful (and often equivalent) ways for depicting and analyzing patterns of change.

Section 12, Habits of Mind.
By the end of 12th grade, students should be able to

- **12B Computation and Estimation:** Use computer spreadsheet, graphing, and database programs to assist in quantitative analysis.

- **12B Computation and Estimation:** Compare data for two groups by representing their averages and spreads graphically.
- **12C Manipulation and Observation:** Learn quickly the proper use of new instruments by following instructions in manuals or by taking instructions from an experienced user.
- **12C Manipulation and Observation:** Use computer for producing tables and graphs and for making spreadsheet calculations.
- **12D Communication Skills:** Choose appropriate summary statistics to describe group differences, always indicating the spread of data as well as the data's central tendencies.
- **12D Communication Skills:** Participate in group discussions on scientific topics by restating or summarizing accurately what others have said, asking for clarification or elaboration, and expressing alternative positions.
- **12D Communication Skills:** Use tables, charts, and graphs in making arguments and claims in oral and written presentations.

Overview:

This three-day lesson will allow students to enhance laboratory technique, as well as familiarize them with using software (such as Excel) to manipulate data, create graphs, and interpret results. Students will perform two investigations concerning biomass conversion to ethanol. Students are expected to use a scientific method in order to create their own scientific investigation.

Purpose:

The purpose of this lesson is three-fold:

- To introduce students to the industrial uses of metabolic pathways, especially biomass conversion using fermentation.
- To encourage students to use a scientific method in order to create their own scientific investigation.
- To enhance laboratory technique and introduce data analysis using computer software (such as Microsoft Excel).

Learning Objectives:

- Students will be introduced to the industrial uses of metabolic pathways.
- Students will have a better understanding of the fermentation process and its industrial use for biomass conversion.
- Students will be able to use the scientific method to create their own scientific investigations.
- Students will be able to use computer software (Microsoft Excel) to create spreadsheets for data, as well as graphs (including appropriate equations and statistical calculations).
- Students will be able to correctly interpret data, and to use charts and graphs to communicate their findings to others.
- Students will be able to compare data sets and draw educated conclusions about causes of variation.

Vocabulary:

Amylase	Enzyme	Non-renewable Energy
Anaerobic	Ethanol	Renewable Energy
Assay	Fermentation	Spectrometer
Biomass	Fructose	Spreadsheet
Buffer	Glucose	Starch
Centrifuge	Metabolic Pathway	Sucrose
Cuvette	Microsoft Excel	Yeast Media

Materials:

Fermentation Materials:

- **Safety glasses**
- Cornstarch (or soluble potato starch)
- Table sugar (sucrose)
- Fructose
- Glucose (dextrose)
- Peptone
- Yeast extract
- Baker's yeast
- Other yeast varieties
- Amylase enzymes:
  - Maxamly from Gist-brocades
  - Amyloglucosidase from Sigma Diagnostics (A 7255)
  - Alpha Amylase from Sigma Diagnostics (A 6211)
- Deionized water
- Stirring rod
- Hot plate
- Thermometer
- Autoclave or pressure cooker
- 125 mL Erlenmeyer flask
- Rubber stopper with hole and tube
- Pipette (including ones that can measure in microliters – may be substituted for a syringe that can accurately measure in microliters)
- Graduated cylinder
- Balance that can accurately weigh to the hundredths of a gram
- Centrifuge
- Parafilm
- Stir plate and stir bar
- Grease pen

Ethanol Assay Materials:

- **Safety glasses**
- **Gloves**
- Ethanol assay kit from Sigma Diagnostics (catalog number 332-A)
  - NAD-ADH Single Assay Vial (individual catalog number 330-1)
  - Ethanol Standard Set (individual catalog number 332-11)
  - Glycine Buffer Reagent (individual catalog number 332-9)
- Spectrometer that can read at 340 nm
- Cuvettes or tubes appropriate for the spectrometer
- Kimwipes
- Saline
- Syringe and needle

Carbon Dioxide Analysis Materials:

- **Safety glasses**
- **Gloves**
- Rubber tubing
- Ring stand and clamps
- 50 mL Burette or Pipette with Stopper
- Bromthymol Blue *(alternative spelling Bromothymol)*
- Tube (minimum one liter)

Data Analysis Materials:

- Computer with Microsoft Excel
- Printer
- Disks for data storage

Preparatory Activities (One Day):

Previous Knowledge and Lessons:

- At this point in the semester, students should already understand the scientific method. In addition, students should know general laboratory procedures, such as taking measurements, data collecting, and record keeping. Although this laboratory will strengthen the understanding of the scientific method, it should not be a new concept and this should not be the first time students are asked to use a scientific method for their laboratory investigations.

- Students should have an introduction to Microsoft Excel before performing the Data Analysis section of this activity. Students should be introduced to the concept of using a spreadsheet and how to convert Excel spreadsheets into graphs.

- This activity is meant to be part of a unit on metabolic pathways. It is best done in conjunction with lessons surrounding glycolysis, cellular respiration, Krebs (TCA) cycle, fermentation (anaerobic respiration), and photosynthesis. Students should understand the big concepts behind these and other metabolic pathways, especially those surrounding fermentation. This activity should enhance students' understanding of the industrial uses of metabolic pathways, with a focus on biomass conversion (fermentation of corn to ethanol).

Day One - Introduction and Laboratory Preparation

Part One - Introduction

15 minutes

- Review of metabolic pathways with an emphasis on fermentation and anaerobic respiration.

30 minutes

- Class discussion: How do we use the metabolic pathways of other organisms (especially microorganisms like bacteria and yeast)? Answers may be written on an overhead, the board, or in student notebooks. The teacher should facilitate this discussion by guiding students to appropriate answers.
- Discussion should be wrapped-up with an emphasis on biomass conversion. This is a good lead-in to the laboratory investigation.
- At times, the teacher should suggest a few uses that the students may not think of or elaborate on student answers….

Food industry
- beer, wine, root beer
- vinegar
- yogurt, cottage cheese, cheese, custard, butter
- sauerkraut
- breads
- sausage, pepperoni, salami
- uses yeast, bacteria
- uses enzymes:
  - chymosin for cheese production
  - amylase to break down starch
  - glucose isomerase to get sweeter products
  - pectinase to clarify fruit juices
  - glucose oxidase to dry egg whites

Drug industry
- organism produces drug as a by-product of metabolic functions
- antibiotics (Penecillin)
- vitamins (A, B2, B12, Biotin, C)

Chemical Industry
- acids (lactic acid, acetic acid)
- alcohols (ethanol)
- others (cellobiose, glucose, xylose, arabinose, xylitol, glycerol)

Symbiotic relationships
- digestion
- lactose-intolerance

Biomass conversion
- plant matter
- **corn to ethanol (LEAD IN TO LAB EXPERIMENT)**

Part Two – Laboratory Preparation

5 minutes
- Overview of three-day laboratory investigation. Remind students that on the third day they will be meeting in the computer lab for data analysis.
- Hand out instructions for the laboratory.
- Review vocabulary as needed.

40 minutes
- Students work in pairs and follow instructions for the first fermentation set-up.
- Students work in pairs to design their own fermentation investigation using a scientific method. Before the fermentation is set-up students report hypothesis and variables to instructor for verification.
- Students keep notes on experiment design in laboratory notebooks and answer questions in the laboratory hand-out.

Main Activities:

Day Two – Identification and Quantification of Fermentation Products

Introduction

25 minutes
- Remind students that on the following day they will be meeting in the computer lab for data analysis.
- Warm-up activity: Ask students to write the chemical equation for the fermentation reaction they set-up the previous day. As a class, go over the reaction and allow students to brainstorm ways they could identify the products. Ask how the products might be quantified.
- Brief review of the carbon dioxide test and the ethanol endpoint assay. If students have not used a spectrometer or a centrifuge before, a brief review on how to use the device may be needed.
- Review vocabulary as needed.

Part One – Carbon Dioxide Analysis

20 minutes

- Students confirm the presence of carbon dioxide as a product in both fermentation reactions (using Bromthymol Blue).
- Students quantify the carbon dioxide production and make quick comparisons and generalizations between both fermentation reactions (using water displacement).
- Students record data in lab notebooks and answer the questions in the lab hand-out.

Part Two – Ethanol Analysis

45 minutes

- Students run the ethanol standards and record data in lab notebooks.
- Students confirm the presence of ethanol and quantify it for each fermentation reaction (using ethanol assay – blood alcohol kit from Sigma Diagnosis).
- Students record data in lab notebooks and answer the questions in the lab hand-out.

Day Three – Data Analysis and Manipulation

Introduction

10 minutes

- Quick review of the day's activities.
- Review vocabulary and Microsoft Excel commands as needed.

Part One – Data Analysis and Manipulation

55 minutes

- Students use Microsoft Excel to create a spreadsheet for the data.
- Students use Microsoft Excel to create graphs for data interpretation.
- Students extrapolate data according to calculations.
- Students write in lab notebook: conclusions (what did they discover about their original hypothesis); ideas for further investigations – what other hypotheses can be made and how can they be tested?

Part Two – Class Discussion (Wrap-up)

25 minutes

- Students share their own investigations as well as their findings from the data analysis. The class discusses general conclusions about the fermentation process and what variables affect the quantity of ethanol and carbon dioxide production.
- Students share ideas for further investigations.

Extensions:

- Students may research the industrial uses of metabolic pathways of other organisms (see list from preparatory activities).
- Websites with experiments on food production using fermentation:
  http://www.uwrf.edu/biotech/workshop/activity/act1/act1.htm
  http://www.lcsc.edu/ns172/Outlines/fermenthome.html
  http://www.wsu.edu:8080/~hurlbert/pages/101lab16.html
  http://www.inform.umd.edu:8080/EdRes/Topic/AgrEnv/ndd/4h/
- Allow students to perform further investigations based on answers to laboratory questions.
- Talk about the uses of the ethanol assay for testing blood-alcohol levels.

Note: Additional attachments include:

- *Teacher Version of Laboratory Procedures*
- *Student Version of Laboratory Procedures*
- *Teacher Version of Data Analysis Procedures*
- *Student Version of Data Analysis Procedures*
- *Assessment Rubric*

A Scientific Investigation on
Alcohol Fermentation and Biomass Conversion
(Teacher Version)

Introduction:

The metabolic pathways of microorganisms produce materials that are useful to mankind. For example, yeast fermentation of simple sugars, such as glucose (dextrose), fructose, and sucrose (table sugar), produce ethanol. Ethanol is an important resource because it can be used as a substitute for gasoline in cars. More importantly, unlike gasoline, ethanol is a renewable energy resource. In industry, ethanol is produced from corn stover (the stalks and leaves of corn plants – *Zea mays*). Other feedstock materials are also being studied as candidates, including wood, other agricultural residues, and municipal solid waste.

During this three-day scientific investigation you will use baker's yeast (*Saccharomyces cerevisiae*) to ferment cornstarch and simple sugars into ethanol. However, yeast cannot breakdown cornstarch alone. As you have learned in previous lessons, many metabolic pathways require specific enzymes. In our case, we need enzymes to break down starch (a polymer made from glucose) into simple sugars.

During this scientific investigation, you will be asked to run two fermentations. You will be asked to use a scientific method to form a hypothesis and develop a laboratory procedure for your own experiment. For example, you may want to vary the type of enzyme used, concentration of reactants, temperature, or other variables you may come up with on your own. You will compare your experiment to a standard procedure and determine the effect(s) on the fermentation products.

Notes for the Instructor:

- *Groups of two or four are suggested, depending on class size and availability of supplies.*
- *Some of the questions provided are challenging. In order to assist students with the more challenging questions, the instructor may opt to provide supplements such as text, MSDS sheets, or original containers. This is a good opportunity to show students how to read and understand MSDS sheets as well as bottle labels.*
- *Allow students to come up with their own investigations. Perhaps they want to try to produce more ethanol, or simply experiment with specific variables. Suggested variables include:*
  - *Starch or sugar concentration*
  - *Enzyme type or concentration*
  - *Yeast type or concentration*
  - *Temperature*
  - *pH of solution*
  - *Other variables students invent*

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Centrifuge	Metabolic Pathway	Sucrose
Cuvette	Microsoft Excel	Yeast Media

Pre-lab Questions:

Please answer the following questions in your lab notebook:

1.) What is the difference between glucose (dextrose), fructose, and sucrose (table sugar)?
2.) Fermentation is what kind of respiration?
3.) Write a balanced equation for the fermentation of glucose to ethanol.
4.) What are the advantages of using ethanol as opposed to gasoline?
5.) Define the vocabulary terms listed above.

Materials and Equipment:

Fermentation Materials:

- **Safety glasses**
- Cornstarch (or soluble potato starch)
- Table sugar (sucrose)
- Fructose
- Glucose (dextrose)
- Peptone

- Yeast extract
- Baker's yeast
- Other yeast varieties
- Amylase and other enzymes
  - Maxamly from Gist-brocades
  - Amyloglucosidase from Sigma Diagnostics (A 7255)
  - Alpha Amylase from Sigma Diagnostics (A 6211)
- Deionized water
- Stirring rod
- Hot plate
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- **Safety glasses**
- **Gloves**
- Rubber tubing
- Ring stand and clamps
- 50 mL Burette or Pipette with Stopper
- Bromthymol Blue *(alternative spelling Bromothymol)*
- Tube (minimum one liter)

Data Analysis Materials:

- Computer with Microsoft Excel
- Printer
- Disks for data storage
- *Note: Data analysis instructions provided separately*

Procedure:

Notes for the Instructor:

Day Zero: Pre-Laboratory Preparation

Before the experiment, the instructor should prepare sterile YP (yeast extract-peptone media) sugar/starch solutions. The solutions should contain 1% yeast extract, 2% peptone, and 0.5% sugar/starch (50 mL for each fermentation will be needed). It is recommended that various sugars (glucose, fructose, sucrose) and concentrations be provided to the students as additional variables for their scientific investigations. Remember that if the sugar concentration is doubled, the ethanol concentration may double as well. In order to quantify the ethanol, it may be necessary to dilute it with saline for the ethanol assay.

In order for the starch to suspend, it is recommended to use soluble potato starch (available from laboratory suppliers) as a substitute for cornstarch. The sugars may be bought at a grocery store, however PURE sugars can be obtained from laboratory suppliers.

Amyloglucosidase and alpha-amylase are available from Sigma-Diagnostics in a powdered form. The following preparations are recommended:

- *Amyloglucosidase (1 unit = 1 mg glucose from starch in 3 minutes at pH 4.5 and 55 ^{o}C; 20,300 units per gram of solid) Dissolve 0.25 grams in 5 mL of deionized, sterile water (adjust according to class size)*
- *Alpha-amylase (1 unit = 1 mg maltose from starch in 3 minutes at pH 6.9 at 20 ^{o}C; 53,000 units per gram of solid) Dissolve 0.1 grams in 5 mL of deionized, sterile water (adjust according to class size)*

Bromthymol Blue comes in a powdered form. Follow the directions on the bottle to make a .5 - 1 liter solution per fermentation. Bromthymol Blue is a pH indicator. It is blue around pH 7.6, green around pH 7, and yellow around pH 6. Add one molar NaOH or HCl, dropwise until Bromthymol Blue turns just blue. It will react with the CO_2 in the air over time, but if used at the correct pH students should see a color change to yellow in the burette/pipette, and only a slight color change in the tube. Remember that the Bromthymol Blue can be reused, the pH simply needs to be adjusted. Provide containers for students to store the Bromthymol Blue after the experiment.

To sterilize the Erlenmeyer flasks, deionized water, and YP sugar/starch solutions, an autoclave should be used (suggested temperature and time: 20 minutes at 121 ^{o}C). A

large home canning pressure cooker may be used as a substitute for the autoclave. PLEASE NOTE that it is IMPORTANT to STERILIZE flasks and solutions in order to prevent unwanted microorganisms from CONTAMINATING the fermentations.

Note: To prevent gelling and clumping of starch/sugar, it is best to make stock solutions of starch, sugar, and YP separately for the autoclave. Mix them together only after they have cooled. Be sure that the concentration of starch/sugar is 0.5% in the final YP-starch/sugar stock solution.

DANGER: When sterilizing liquids under high pressure and temperature, the containers MUST be vented to prevent EXPLOSION! Please refer to the SAFETY INSTRUCTIONS provided with your autoclave or pressure cooker!

Day One – Fermentation Set-up

Today you will be setting up two fermentations. The fermentations will run over-night and the following day you will identify and quantify the products. Your instructor has already prepared sterile YP (yeast extract-peptone) media necessary for a stable yeast culture.

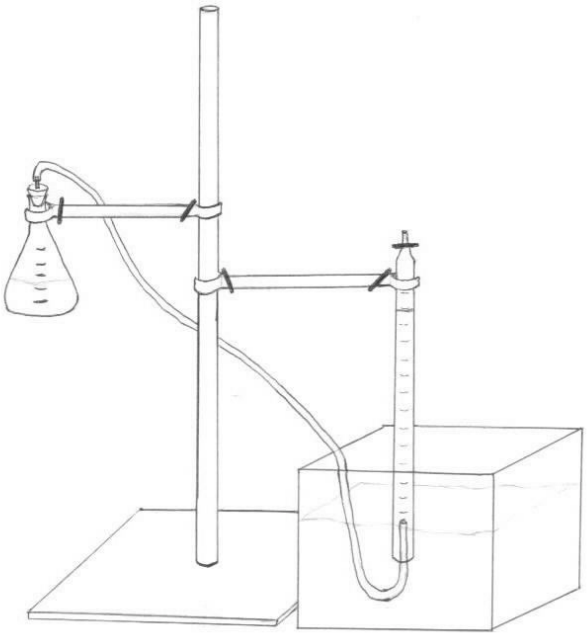
Throughout your scientific investigation remember to use good laboratory technique. This includes taking good notes in your lab notebook! This means that if someone were to read your lab notebook they should be able to understand what you did. In addition, your notes should be neat and detailed enough that another person could reproduce your experiment! Do not forget to note things like color, consistency, bubbles, measurements, spills, etc. Also include any questions or ideas that come up while you are performing your experiment.

WEAR SAFETY GLASSES AT ALL TIMES – STUDENTS CAUGHT WITHOUT SAFETY GLASSES WILL IMMEDIATLY BE REMOVED FROM THE LAB AND GIVEN A 0 FOR THIS EXPERIMENT!!!

Fermentation One:

1.) Obtain all the necessary laboratory equipment
2.) Pipette 50 mL of 0.5% YP-starch solution to a sterile, 125 mL Erlenmeyer flask (be sure the solution is being stirred while you pipette – this is best done on an electric stir plate)
3.) Add 1 mL of Amyloglucosidase enzyme
4.) Add a pinch of bakers yeast (note the time in your lab notebook)
5.) Cap the flask with a rubber stopper that contains a hole with a tube extending out of the stopper
6.) Swirl the solution to mix the contents
7.) WEAR GLOVES FOR THIS NEXT PART – YOU DO NOT WANT TO STAIN YOUR HANDS!!!
8.) Set-up the CO2 identification and quantification apparatus as shown in the diagram below...

- Attach a rubber hose to the tube extending out of the stopper
- Mount the Erlenmeyer flask on your ring stand
- Fill a burette or pipette with Bromthymol Blue (be sure that the other end is stoppered before you do this step)
- Pour the rest of the Bromthymol Blue into the tube
- Carefully invert the burette/pipette into the tube, trying not to let too much air into the burette/pipette
- Clamp the burette/pipette to the ring stand
- Insert the rubber tubing from the Erlenmeyer flask into the bottom of the burette/pipette
- With a grease pen, mark the meniscus of the Bromthymol Blue (note the color and mL marking in your lab notebook)



Fermentation Two:

For the second fermentation you will be using a scientific method to develop your own experiment. Use the following questions to help you design your scientific investigation. Be sure to keep good notes in your lab notebook (answering the following questions will help you to do this)!

- Invent a question about the first fermentation. Be sure this question can be answered through an experiment. (Example: What happens when the fermentation is performed at 50 ^{0}C?)
- What is your hypothesis?
- Design a way to test this hypothesis.
- What variables will you control?

- What variable will you change?
- What would a positive test look like?
- What would a negative test look like?

Note: Do not forget to mark your Erlenmeyer flasks so they can be easily identified! You should note this in your lab notebook.

Day One Questions:

Answer the following questions in your lab notebook:

1.) Why does the yeast need sterile YP media?
2.) What do you think the yeast-extract may contain? Why might the yeast need this?
3.) What do you think the peptone may contain? Why might the yeast need this?
4.) Why does the YP-starch solution need to be stirred while you pipette it?
5.) What is Amyloglucosidase and why do we need it?
6.) What is Bromthymol Blue and why do you think we are using it?
7.) What is the purpose of the inverted burette/pipette?
8.) Predict the products from both of your fermentations. How might they might be different?
9.) Predict the amount of CO_2 produced for both of your fermentations.
10.) Predict the amount of ethanol produced for both of your fermentations.

Day Two – Product Identification and Quantification

Today you will be confirming the presence of CO_2 and ethanol, as well as quantifying these products. In order to quantify the ethanol production, you will be using a blood-alcohol kit. Yes, this is the same kit that may be used to test the alcohol content in your blood or urine. However, we are going to use it to test the ethanol produced in a fermentation! The reaction takes place as follows:

$$\text{Ethanol} + \text{NAD} \xrightarrow{\text{ADH}} \text{acetaldehyde} + \text{NADH}$$

(Note: ADH = alcohol dehydrogenase)

As NAD is converted into NADH, the absorbance at 340 nm increases. Thus, the increase in absorbance is directly proportional to the alcohol concentration. If you test know concentrations of ethanol, you will be able to create a standard curve. This will give you something to compare your values to so that you can determine the ethanol concentration of your fermentation.

Notes for the Instructor:

- When the ethanol assay kit arrives, remember to keep the NAD-ADH Single Assay Vials frozen. However, remember to remove them in time for the vials to thaw before the students arrive.
- The instructor should make a 'blank' for the spectrometer. This should be done the same way students are doing the ethanol assay, simply substitute 10 microliters of deionized water for 10 microliters of sample.
- The instructor should also prepare one set of ethanol standards around the same time as the first group is ready to use the spectrometer.
- Be sure that students confirm that they are aware of the need to wear safety glasses and gloves while handling Glycine Buffer Reagent.

DANGER: Glycine Buffer Reagent is TOXIC! It is very IMPORTANT that everyone wear GLOVES during this experiment. SENSITIZATION to Glycine Bluffer Reagent is caused by INHALATION and SKIN CONTACT.

WEAR SAFETY GLASSES AND GLOVES AT ALL TIMES – STUDENTS CAUGHT WITHOUT SAFETY GLASSES AND GLOVES WILL IMMEDIATLY BE REMOVED FROM THE LAB AND GIVEN A 0 FOR THIS EXPERIMENT!!!

CO_2 Identification and Quantification

1.) Examine the color of the Bromthymol Blue in the burette/pipette. Record your observations in your lab notebook
2.) Using a grease pen, mark the location of the bottom of the meniscus
3.) Compare the initial meniscus line to the current meniscus line.

Ethanol Identification and Quantification

1.) Obtain two NAD-ADH Single Assay Vials from your instructor
2.) Unstopper your Erlenmeyer flask and quickly pipette 1 mL of your fermentation sample into a test-tube and cover with parafilm ASAP (be sure to re-stopper your flask). **Be sure not to disturb the sediment on the bottom of your flask** (only pipette from the top portion)
3.) Since you have two fermentations, you should be able to balance the centrifuge with your own test-tubes

IMPORTANT NOTE: DO NOT run the centrifuge until it has been CORRECTLY BALANCED. CHECK with your INSTRUCTOR before turning on the CENTRIFUGE!

4.) Spin your samples at 1000 rpm for 10 minutes
5.) While your samples are spinning, obtain Glycine Buffer Reagent from your instructor. **Before your instructor gives you the Glycine, make it clear to your**

instructor that you understand the need for safety glasses and gloves while handling this chemical.

6.) Add 3.0 mL of Glycine Buffer Reagent to each of your NAD-ADH Single Assay Vials
7.) Cap and invert gently several times in order to dissolve the powder **– DO NOT SHAKE VIAL!!!**
8.) After your samples are removed from the centrifuge, add 0.01 mL (10 microliters) of your fermentation sample to each vial (record the time in your lab notebook).
9.) Be sure to recap your vials and invert gently to mix – **DO NOT SHAKE VIAL!!!**
10.) Allow your samples to incubate for 10 minutes (be sure the temperature is between 22 ^{o}C and 37 ^{o}C)
11.) Obtain cuvettes from your instructor. **Remember that the oils on your hands will interfere with the spectrometer. Only handle the cuvettes on the frosted side and be sure to use kimwipes to clean smudges.**
12.) Zero the spectrometer using the blank your instructor prepared for you
13.) Measure the absorbance of the ethanol standards that your instructor prepared for you (remember that there are other groups waiting to use the spectrometer, so please be efficient)
14.) Measure the absorbance for your samples

Note: Be sure that you are labeling all of your vials, test tubes, etc. so that you can easily identify them (note this in your lab notebook).

Day Two Questions:

Answer the following questions in your lab notebook:

1.) How did the Bromthymol Blue change and what does it mean?
2.) What does the comparison of the two meniscus lines tell you about the CO_2 production?
3.) When you compare the CO_2 production from both of your fermentations, what does this tell you about your experiment?
4.) Make another prediction about the ethanol production now that you know the CO_2 production.
5.) Where have you seen NAD and NADH in metabolic pathways?
6.) Is the NAD -> NADH a reduction or an oxidation reaction?
7.) What is a spectrometer and how does it work?
8.) What type of light is at 340 nm? What is the range for the visible spectrum?
9.) Why is it important to zero the spectrometer?
10.) Why do we need to measure the aborbance of ethanol standards?
11.) Why is it so important to keep your samples covered with parafilm?
12.) Why is it important to balance the centrifuge?
13.) What is a centrifuge and why do we need to use it?

A Scientific Investigation on Alcohol Fermentation and Biomass Conversion

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Centrifuge	Metabolic Pathway	Sucrose
Cuvette	Microsoft Excel	Yeast Media

Pre-lab Questions:

Please answer the following questions in your lab notebook:

1.) What is the difference between glucose (dextrose), fructose, and sucrose (table sugar)?
2.) Fermentation is what kind of respiration?
3.) Write a balanced equation for the fermentation of glucose to ethanol.
4.) What are the advantages of using ethanol as opposed to gasoline?
5.) Define the vocabulary terms listed above.

Materials and Equipment:

Fermentation Materials:

- **Safety glasses**
- Cornstarch (or soluble potato starch)
- Table sugar (sucrose)
- Fructose
- Glucose (dextrose)
- Peptone
- Yeast extract
- Baker's yeast
- Other yeast varieties
- Amylase and other enzymes
  - Maxamly from Gist-brocades
  - Amyloglucosidase from Sigma Diagnostics (A 7255)
  - Alpha Amylase from Sigma Diagnostics (A 6211)
- Deionized water
- Stirring rod
- Hot plate
- Thermometer
- Autoclave or pressure cooker
- 125 mL Erlenmeyer flask
- Rubber stopper with hole and tube
- Pipette (including ones that can measure in microliters – may be substituted for a syringe that can accurately measure in microliters)
- Graduated cylinder
- Balance that can accurately weigh to the hundredths of a gram
- Centrifuge
- Parafilm
- Stir plate and stir bar
- Grease pen

Ethanol Assay Materials:

- **Safety glasses**
- **Gloves**
- Ethanol assay kit from Sigma Diagnostics (catalog number 332-A)
  - NAD-ADH Single Assay Vial (individual catalog number 330-1)
  - Ethanol Standard Set (individual catalog number 332-11)
  - Glycine Buffer Reagent (individual catalog number 332-9)
- Spectrometer that can read at 340 nm
- Cuvettes or tubes appropriate for the spectrometer
- Kimwipes
- Saline
- Syringe and needle

Carbon Dioxide Analysis Materials:

- **Safety glasses**
- **Gloves**
- Rubber tubing
- Ring stand and clamps
- 50 mL Burette or Pipette with Stopper
- Bromthymol Blue *(alternative spelling Bromothymol)*
- Tube (minimum one liter)

Data Analysis Materials:

- Computer with Microsoft Excel
- Printer
- Disks for data storage
- *Note: Data analysis instructions provided separately*

Procedure:

Day One – Fermentation Set-up

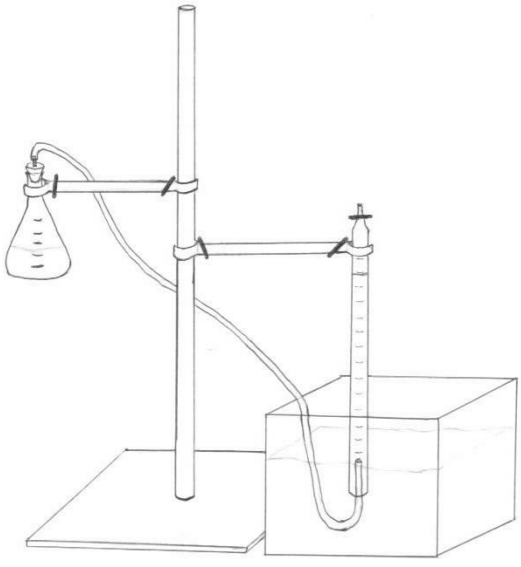
Today you will be setting up two fermentations. The fermentations will run overnight and the following day you will identify and quantify the products. Your instructor has already prepared sterile YP (yeast extract-peptone) media necessary for a stable yeast culture.

Throughout your scientific investigation remember to use good laboratory technique. This includes taking good notes in your lab notebook! This means that if someone were to read your lab notebook they should be able to understand what you did. In addition, your notes should be neat and detailed enough that another person could reproduce your experiment! Do not forget to note things like color, consistency, bubbles, measurements, spills, etc. Also include any questions or ideas that come up while you are performing your experiment.

WEAR SAFETY GLASSES AT ALL TIMES – STUDENTS CAUGHT WITHOUT SAFETY GLASSES WILL IMMEDIATLY BE REMOVED FROM THE LAB AND GIVEN A 0 FOR THIS EXPERIMENT!!!

Fermentation One:

1.) Obtain all the necessary laboratory equipment
2.) Pipette 50 mL of 0.5% YP-starch solution to a sterile, 125 mL Erlenmeyer flask (be sure the solution is being stirred while you pipette – this is best done on an electric stir plate)
3.) Add 1 mL of Amyloglucosidase enzyme
4.) Add a pinch of bakers yeast (note the time in your lab notebook)
5.) Cap the flask with a rubber stopper that contains a hole with a tube extending out of the stopper
6.) Swirl the solution to mix the contents
7.) WEAR GLOVES FOR THIS NEXT PART – YOU DO NOT WANT TO STAIN YOUR HANDS!!!
8.) Set-up the CO2 identification and quantification apparatus as shown in the diagram below...
    - Attach a rubber hose to the tube extending out of the stopper
    - Mount the Erlenmeyer flask on your ring stand
    - Fill a burette or pipette with Bromthymol Blue (be sure that the other end is stoppered before you do this step)
    - Pour the rest of the Bromthymol Blue into the tube
    - Carefully invert the burette/pipette into the tube, trying not to let too much air into the burette/pipette
    - Clamp the burette/pipette to the ring stand
    - Insert the rubber tubing from the Erlenmeyer flask into the bottom of the burette/pipette
    - With a grease pen, mark the meniscus of the Bromthymol Blue (note the color and mL marking in your lab notebook)



Fermentation Two:

For the second fermentation you will be using a scientific method to develop your own experiment. Use the following questions to help you design your scientific investigation. Be sure to keep good notes in your lab notebook (answering the following questions will help you to do this)!

- Invent a question about the first fermentation. Be sure this question can be answered through an experiment. (Example: What happens when the fermentation is performed at 50 ^{0}C?)
- What is your hypothesis?
- Design a way to test this hypothesis.
- What variables will you control?
- What variable will you change?
- What would a positive test look like?
- What would a negative test look like?

Note: Do not forget to mark your Erlenmeyer flasks so they can be easily identified! You should note this in your lab notebook.

Day One Questions:

Answer the following questions in your lab notebook:

1.) Why does the yeast need sterile YP media?
2.) What do you think the yeast-extract may contain? Why might the yeast need this?
3.) What do you think the peptone may contain? Why might the yeast need this?
4.) Why does the YP-starch solution need to be stirred while you pipette it?
5.) What is Amyloglucosidase and why do we need it?
6.) What is Bromthymol Blue and why do you think we are using it?
7.) What is the purpose of the inverted burette/pipette?
8.) Predict the products from both of your fermentations. How might they might be different?
9.) Predict the amount of CO_2 produced for both of your fermentations.
10.) Predict the amount of ethanol produced for both of your fermentations.

Day Two – Product Identification and Quantification

Today you will be confirming the presence of CO_2 and ethanol, as well as quantifying these products. In order to quantify the ethanol production, you will be using a blood-alcohol kit. Yes, this is the same kit that may be used to test the alcohol content in your blood or urine. However, we are going to use it to test the ethanol produced in a fermentation! The reaction takes place as follows:

Ethanol + NAD $\xrightarrow{\text{ADH}}$ acetaldehyde + NADH
(Note: ADH = alcohol dehydrogenase)

As NAD is converted into NADH, the absorbance at 340 nm increases. Thus, the increase in absorbance is directly proportional to the alcohol concentration. If you test know concentrations of ethanol, you will be able to create a standard curve. This will give you something to compare your values to so that you can determine the ethanol concentration of your fermentation.

DANGER: Glycine Buffer Reagent is TOXIC! It is very IMPORTANT that everyone wear GLOVES during this experiment. SENSITIZATION to Glycine Bluffer Reagent is caused by INHALATION and SKIN CONTACT.

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CO_2 Identification and Quantification

1.) Examine the color of the Bromthymol Blue in the burette/pipette. Record your observations in your lab notebook
2.) Using a grease pen, mark the location of the bottom of the meniscus
3.) Compare the initial meniscus line to the current meniscus line.

Ethanol Identification and Quantification

1.) Obtain two NAD-ADH Single Assay Vials from your instructor
2.) Unstopper your Erlenmeyer flask and quickly pipette 1 mL of your fermentation sample into a test-tube and cover with parafilm ASAP (be sure to re-stopper your flask). **Be sure not to disturb the sediment on the bottom of your flask** (only pipette from the top portion)
3.) Since you have two fermentations, you should be able to balance the centrifuge with your own test-tubes

IMPORTANT NOTE: DO NOT run the centrifuge until it has been CORRECTLY BALANCED. CHECK with your INSTRUCTOR before turning on the CENTRIFUGE!

4.) Spin your samples at 1000 rpm for 10 minutes
5.) While your samples are spinning, obtain Glycine Buffer Reagent from your instructor. **Before your instructor gives you the Glycine, make it clear to your instructor that you understand the need for safety glasses and gloves while handling this chemical.**

6.) Add 3.0 mL of Glycine Buffer Reagent to each of your NAD-ADH Single Assay Vials
7.) Cap and invert gently several times in order to dissolve the powder **– DO NOT SHAKE VIAL!!!**
8.) After your samples are removed from the centrifuge, add 0.01 mL (10 microliters) of your fermentation sample to each vial (record the time in your lab notebook).
9.) Be sure to recap your vials and invert gently to mix – **DO NOT SHAKE VIAL!!!**
10.) Allow your samples to incubate for 10 minutes (be sure the temperature is between 22 ^{o}C and 37 ^{o}C)
11.) Obtain cuvettes from your instructor. **Remember that the oils on your hands will interfere with the spectrometer. Only handle the cuvettes on the frosted side and be sure to use kimwipes to clean smudges.**
12.) Zero the spectrometer using the blank your instructor prepared for you
13.) Measure the absorbance of the ethanol standards that your instructor prepared for you (remember that there are other groups waiting to use the spectrometer, so please be efficient)
14.) Measure the absorbance for your samples

Note: Be sure that you are labeling all of your vials, test tubes, etc. so that you can easily identify them (note this in your lab notebook).

Day Two Questions:

Answer the following questions in your lab notebook:

1.) How did the Bromthymol Blue change and what does it mean?
2.) What does the comparison of the two meniscus lines tell you about the CO_2 production?
3.) When you compare the CO_2 production from both of your fermentations, what does this tell you about your experiment?
4.) Make another prediction about the ethanol production now that you know the CO_2 production.
5.) Where have you seen NAD and NADH in metabolic pathways?
6.) Is the NAD -> NADH a reduction or an oxidation reaction?
7.) What is a spectrometer and how does it work?
8.) What type of light is at 340 nm? What is the range for the visible spectrum?
9.) Why is it important to zero the spectrometer?
10.) Why do we need to measure the aborbance of ethanol standards?
11.) Why is it so important to keep your samples covered with parafilm?
12.) Why is it important to balance the centrifuge?
13.) What is a centrifuge and why do we need to use it?

Data Analysis Using Excel
(Teacher Version)

A major component of any scientific investigation is the data analysis. Often times scientists use computer software to help them interpret data. Today you will use Microsoft Excel to help you analyze the data from your scientific investigation. This lesson will help you learn how to use Excel. Keep in mind that commands may vary from version-to-version. Ask your instructor for help or use the help tool if you run into any problems.

Notes to the Instructor:

This packet was put together with commands from Microsoft Excel 97. Other versions may be slightly different. Remember to inform students about any major changes.

In order to analyze your data from the ethanol assay, you need a way to relate the absorbance to ethanol concentrations. To do this you are going to make a standard curve using KNOWN ethanol concentrations. By graphing the absorbance versus the ethanol concentration, you will be able to use Excel to fit an equation to these values. This equation can then be used to calculate the ethanol concentrations for your fermentation samples. Remember that "absorbance vs. ethanol concentration" means that absorbance will be on your y-axis.

Excel Procedures:

WARNING: Remember to SAVE your work OFTEN! If you are not SAVING EVERY 5-10 MINUTES, then you are not saving OFTEN enough. When you are finished for the day, you should back up your files on a floppy disk so that in the future you will be able to go back and edit or print those files.

Part One – The Standard Curve

1.) **Enter your data for the standard curve.** Be sure that your data for the x-axis is in the left column. Remember to label the sets of data so that later you will remember where the numbers came from!
2.) To graph this data, highlight the values you want to appear in the graph. Under the **"Insert"** menu, choose **"Chart..."** At this point you need to decide what type of graph you would like to make. In this case, we want to make a standard curve. Thus, choose the **"XY (Scatter)" plot.** When you highlight this type of plot, a series of choices will appear to the right. Chose the top one without any lines connecting the points. After you choose the correct graph, press the **"Next"** button at the bottom of the screen.

3.) Do not change anything on the next screen. Simply press the **"Next"** button again.
4.) Notice that at the top of this next screen there are multiple tabs. Under the **"Titles"** tab, **enter a title for your graph. Define the x-axis and y-axis** (be sure to include units for concentration).
5.) On the same screen, under the tab **"Gridlines"** there will be a check next to "Major gridlines". **Remove this check** by clicking in the box.
6.) On the same screen, under the tab **"Legend" remove the check by "Show legend."** Click the **"Finish"** button to insert your graph into the spreadsheet.
7.) Notice the gray background on your graph? This wastes ink. To change the background to white **double click inside the graph.** Under the section called **"Area"** check **"None".** Press the **"OK"** button when you are finished.
8.) Examine the points on your graph. In your lab notebook **predict what type of relationship** this is (i.e. logarithmic, exponential, liner, polynomial, etc.)
9.) **Select your graph** by clicking on it. Under the **"Chart"** menu, **choose "Add Trendline..."**
10.) Choose the appropriate **trendline** for your points under the **"Type"** tab. Under the **"Options"** tab, check **"Display equation on chart".** Press the **"OK"** button when you are finished.
11.) You may want to **move your equation** if it is in the way of the graph. In addition, you may want to change the thickness of your trendline so that the points remain clearly defined. To do this double click on the trendline and under the **"Patterns"** tab, choose the **"Weight"** that looks best. Press the **"OK"** button when you are finished.
12.) **Select the graph** by clicking on it. **Print** your graph and **insert** it into your lab notebook.

Part Two – Calculating Ethanol Concentrations for the Fermentations

1.) Select the **"Sheet 2"** tab at the bottom of your screen. This will save the following data table in the same file, but allow you to use a new spreadsheet.
2.) **Enter the absorbencies** for your two fermentations into the spreadsheet. Include a column to **identify** which fermentation is which.
3.) Label another column for your **calculated ethanol concentration. Predict** a way you might do this on your calculator. **Predict** a way you might use Excel to do this. Write your answers in your lab notebook.
4.) Remember the equation on your standard curve graph? Use this equation to make an equation in Excel. Click in the box where you would like **your calculated ethanol concentration** for the first fermentation. **Enter an equation.** For example, if your equation was "y = 5x + 3" the equation you would want to enter in Excel would be =((y – 3) / 5), where y = the box containing the absorbance for the first fermentation (see example below).

	A	B	C
1	Fermentation number	Absorbance	Calculated ethanol concentration
2	1	.326	=((B2 - 3) / 5)
3	2	.065	

5.) To use the same equation in the rest of the column, **highlight** the boxes in the column you are working with. Under the **"Edit"** menu, choose **"Fill..."** and then choose **"Down".** Although today you only have two columns to fill, in the future you may have many. Using the command explained above, you can save yourself a lot of typing!

Questions:

Answer the following questions in your lab notebook:

1.) What is the difference between an independent variable and a dependent variable?
2.) On which axis should the independent variable be graphed? What is the independent variable in your experiment?
3.) Why did we choose the "XY (Scatter)" plot as opposed to one of the other choices?
4.) Why did we choose to plot the graph without connecting the points?
5.) What kind of relationship does the ethanol standard curve show?
6.) What kind of trendline did you use and what was the equation generated?
7.) How are the ethanol concentrations different in your two fermentations? What does this tell you about your scientific investigation?
8.) Was your original hypothesis correct?
9.) Taking your data into consideration, what other scientific investigations would you want to perform in order to understand the effects of the variables?
10.) After sharing your results with your classmates, how has your answer to question nine changed?
11.) After sharing your results with your classmates, design another scientific investigation. Use the same set of questions presented in the laboratory procedures to guide you through the design process.

Notes for the Instructor:

The following pages contain data from an actual experiment. This is meant to give you an idea of what student answers should look like.

Explanation of Fermentation Set-up:

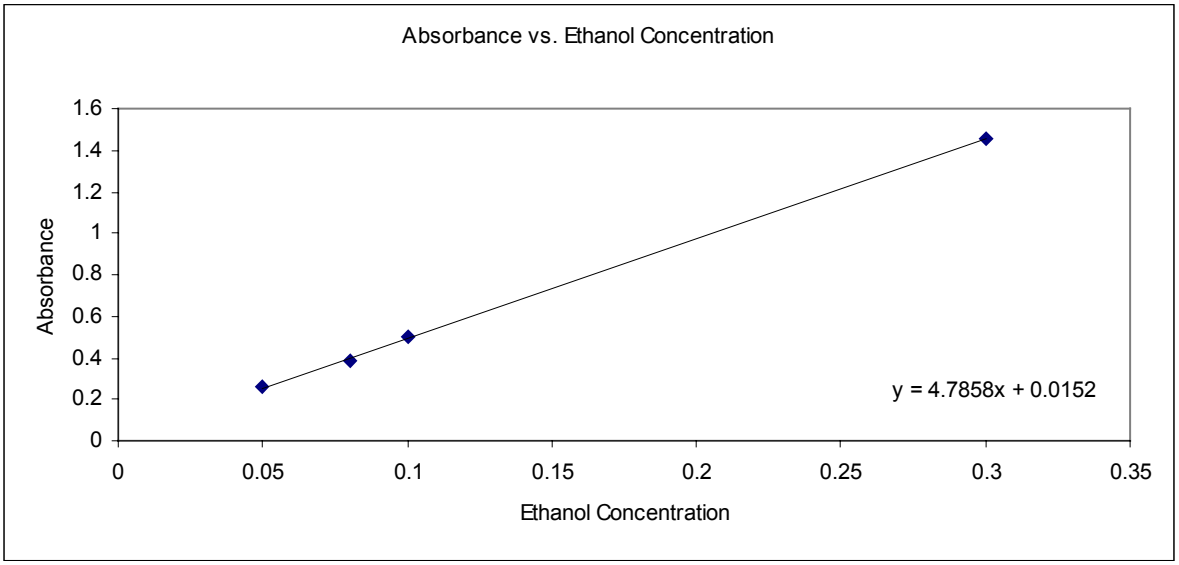
The following fermentations were performed using....

Sample #	Starch/Sugar	Enzyme	Yeast	Yeast extract (1%) Peptone (2%)
A	None	None	Yes	Yes
B	None	Amyloglucosidase (AG)	Yes	Yes
C	None	Alpha-Amylase (aA)	Yes	Yes
D	None	AG & aA	Yes	Yes
E	None	Maxamyl (M)	Yes	Yes
F	1% Glucose	None	Yes	Yes
G	1% Fructose	None	Yes	Yes
H	1% Sucrose	None	Yes	Yes
I	1% Starch	None	Yes	Yes
J	1% Starch	AG	Yes	Yes
K	1% Starch	AA	Yes	Yes
L	1% Starch	AG & aA	Yes	Yes
M	1% Starch	M	Yes	Yes
N	1% Glucose	None	No	Yes

The above fermentations were constructed in order to test the ethanol assay and determine which set-up would be the best for the "teacher-designed" fermentation (or the first fermentation that the students perform). These fermentations determine the background sugar content of the media and the enzymes. Please note that if the absorbance obtained by the students are at a value greater than one, that the assay would need to be re-run after diluting it with saline. Most spectrometers available at a high school are not accurate at values greater than one. Also note that the sugar concentration in these experiments was 1% as opposed to the 0.5% suggested in the laboratory. Below is the standard curve for the ethanol assay, as well as the calculations for the ethanol content of the above fermentations...

Standard Curve:

ethanol concentration	absorbance
0.3	1.4513
0.1	0.4978
0.08	0.3879
0.05	0.2601



absorbance of unknown concentrations	sample number	calculated ethanol concentration
0.1053	A	0.018868319
0.2495	B	0.048999122
0.1013	C	0.018032513
0.2291	D	0.044736512
0.8829	E	0.181348991
2.1565	F	0.447469598
2.2124	G	0.459149985
2.3243	H	0.482531656
0.0507	I	0.007459568
2.1166	J	0.439132433
0.3232	K	0.064398847
1.9044	L	0.394792929
1.4476	M	0.299343892
0.0049	N	-0.00211041
2.2817	Oh!	0.473630323

Notice that the YP media and the enzymes did contain some background sugars (fermentations A-E show this). These remaining fermentations (F-N) are corrected in the following table.

Sample Number	Corrected With...	Corrected Ethanol Concentration
F	A (-0.018868319)	0.428601279
G	A (-0.018868319)	0.440281666
H	A (-0.018868319)	0.463663337
I	A (-0.018868319)	-0.011408751
J	B (-0.048999122)	0.390133311
K	C (-0.018032513)	0.046366334
L	D (-0.044736512)	0.350056417
M	E (0.181348991)	0.117994901
N	A (-0.018868319)	-0.020978729

Notice that fermentation 'I' and 'N' gave negative values for ethanol concentration. Why? Because no ethanol was produced in these fermentations. In the case of fermentation 'I', starch should not be converted to ethanol unless enzymes are present (the enzymes break starch down into simple sugars which the yeast CAN convert). Since no enzymes were added, the fermentation did not occur. In the case of fermentation 'N', no yeast was added. Without this microorganism, fermentation can occur (assuming you have a sterile solution).

Also note that out of the three enzymes, the two which worked the best contained Amyloglucosidase. The Maxamyl enzyme showed some ethanol production, and the production using alpha-amylase showed poor ethanol yields.

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